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(54) NOUVEAUX SYSTEMES DE REGULATION DE L'EXPRESSION
GENETIQUE

(54) NEW SYSTEMS FOR REGULATING GENETIC EXPRESSION

(57) La présente invention concerne un procédé pour sélectionner de nouvelles séquences d'opérateurs P_R ou P_L constituées de phages lambdoïdes qui présentent une thermostabilité différente de celle des séquences de type sauvage, pour la fixation d'un répresseur. L'invention concerne en outre de nouvelles séquences d'opérateurs P_R ou P_L mutées, ainsi que leur utilisation pour l'expression génétique thermorégulée et pour la préparation de vaccins améliorés.

(57) The present invention concerns a process for selecting new P_R - or P_L -operator sequences of lambdoid phages which, compared to wild-type sequences, have a different thermostability for the binding of a repressor. In addition, the invention discloses new mutated PR- or PL-operator sequences and their use for temperature-regulated expression of genes and for producing improved vaccines.



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New systems for the regulation of gene expression

Description

The present invention concerns a method for selecting new P_R or P_L operator sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding a repressor. In addition new mutated P_R or P_L operator sequences and their application for the temperature-regulated expression of genes and for the production of improved vaccines are disclosed.

The initiation of transcription of the O_R-O_L region of the bacteriophage lambda and other lambdoid phages is negatively and positively regulated by a repressor which is the product of the cI gene (see review article Ptashne et al., Cell 19 (1980), 1-11). In the O_R region three operator sequences (O_{R1} , O_{R2} and O_{R3}) overlap the promoters P_R and P_{RM} which are orientated in different directions. P_R controls the transcription of genes which are responsible for the lytic multiplication cycle of the phage whereas P_{RM} is the promoter for the lambda cI gene which is responsible for maintaining the lysogenic state. The lambda repressor cI binds co-operatively to the operator sequences O_{R1} and O_{R2} with the result that P_R is repressed and P_{RM} is activated.

In addition the bacteriophage lambda also contains a further operator region O_L which also contains three operator sequences (O_{L1} , O_{L2} and O_{L3}). The expression of the lambda N gene can be repressed by the P_L promoter by binding of the cI repressor to this O_L operator region.

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Promoters of the bacteriophage lambda in particular the P_L and the P_R promoter have been used for a long time in recombinant DNA technology for heterologous temperature-regulated gene expression in *E.coli* (cf. Hedgpeth et al., Molec.Gen.Genet. 183 (1978), 197-203 and Bernard et al., Gene 5 (1979), 59-76; Buell et al., Nucleic Acids Res. 13 (1985), 1923 and Shatzman and Rosenberg, Methods Enzymol. 152 (1987), 661). A temperature-sensitive lambda repressor cI857 is used in these expression systems which represses the P_L and P_R transcription at low temperatures up to 30°C but allows a gene expression at higher temperatures.

An advantage of this lambda expression system is that the gene expression can be induced in a simple manner by increasing the temperature and no addition of chemical inducers is necessary for this. However, a serious disadvantage is that the repression of gene expression only occurs up to relatively low temperatures of not more than 30°C, which is a temperature at which only a slow bacterial growth occurs. Hence the object of the invention was to provide an improved system for lambda P_L or P_R gene expression which enables a repression at variable higher temperatures.

This object is achieved by providing mutated P_R or P_L operator sequences from lambdoid phages which, compared to the wild-type operator sequence, have a different and in particular higher thermostability with regard to the binding of a temperature-sensitive repressor. The finding that lambda expression systems with an improved thermostability can be produced at all is extremely surprising since, apart from the temperature-sensitive lambda cI857 mutant, no other temperature-sensitive cI mutants are known but only those mutations in the cI

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repressor are known which make the molecule more resistant to thermal inactivation (Hecht et al., Proteins 1 (1986), 43-46 and Das and Mandal, Mol.Gen.Genet. 204 (1986), 540-542). It was even more surprising that mutations which lead to an improved thermostability are located in the operator DNA sequence and not in the DNA sequence coding for the repressor molecule. Thus for example a mutation of the lambda O_R2 operator sequence is known from the literature which leads to a complete loss of repressor binding (Hawley et al., J.Biol.Chem. 260 (1985), 8618-8626).

A method is provided for identifying suitable mutants which enables the selection of mutated O_R or O_L operator DNA sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding a repressor in which the method is characterized in that (a) a DNA cassette is prepared which contains a selection gene under the operative control of an expression control sequence comprising at least one O_R or O_L operator sequence from a lambdoid phage and a promoter, (b) the operator DNA sequence is subjected to a mutagenesis and (c) the mutated operator DNA sequences are analysed.

The lambdoid phages are preferably selected from the group comprising the phage lambda, phage 21, phage 22, phage 82, phage 424, phage 434, phage D326, phage DLP12, phage gamma, phage HK022, phage P4, phage Phi80, phage Phi81, coliphage 186 and recombinant variants thereof. The said phages are very similar with regard to the mechanism of repression of gene expression by means of a cI repressor (Johnson et al., Nature 294 (1982), 217-223). Recombinant variants of the said phages e.g. lambda imm434 can be obtained by substitution of

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individual genome fragments within the said phages (cf. for this Hendricks et al., Lambda 2 (1983), R.W. Hendricks, J.W. Roberts, F.W. Stahl and R.A. Weissberg (publisher), Cold Spring Harbor Laboratory Press, New York). The phage lambda or a recombinant variant thereof is preferably used as the lambdoid phage e.g. lambda imm434. An operator DNA sequence from the operator regions O_R (SEQ ID NO.1) or /and O_L (SEQ ID NO.3) of the phage lambda and in particular one of the operator sequences O_{R1} , O_{R2} and O_{R3} or O_{L1} , O_{L2} and O_{L3} contained therein is particularly preferably used for the mutagenesis. The operator sequence O_R2 is most preferred.

The selection gene for the DNA cassette which is brought under the operative control of the expression control sequence containing the mutated operator sequence, preferably a lambda operator/promoter region, is preferably a suicide gene which when expressed leads to the death of the bacterial cell and thus serves as a selection marker for identifying suitable mutants. The suicide gene should be so strongly repressed at a temperature at which the lambda repressor binds to the mutated operator sequence that a bacterial cell containing the DNA cassette can grow. When the maximum temperature at which the repressor can still bind to the operator is exceeded, the suicide gene is expressed and the bacterial cell is destroyed. This enables a simple and direct selection of suitable mutated operator sequences. A suitable suicide gene is the E lysis gene from the phage PhiX174 as well as homologues and derivatives derived therefrom (Hutchison and Sinsheimer, J.Mol.Biol. 18 (1966), 429-447; Witte et al., Multifunctional safety vector systems for DNA cloning, controlled expression of fusion genes and

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simplified preparation of vector DNA and recombinant gene products, in BioTech Forum, Advances in Molecular Genetics 3, pp 219-239, publisher: Issinger, O.-G., Henke, J., Kämpf, J., Driesel, A.J., Hüthing Verlag 1991, Heidelberg). Further examples of suitable lysis genes are GEF (Poulsen et al., Mol.Microbiol. 5 (1991), 1627-1637) and Kil (Reisinger et al., Virology 193 (1993), 1033-1036). On the other hand the selection gene can also be a reporter gene such as e.g. the β -Gal gene.

In order to produce mutants the operator DNA sequence is preferably subjected to a site-specific mutagenesis using one or several oligonucleotides for example according to the method of Kunkel (Proc.Natl.Acad.Sci. USA 82 (1985), 488-492) or they are obtained by selection in a mutator bacterial strain e.g. an E. coli mutD or mutL mutator strain such as E. coli ES1578 (Wu et al., Gene 87 (1990), 1-5). The mutated operator DNA sequences are preferably selected by determining the ability to bind to a temperature-sensitive cI repressor in particular to the temperature-sensitive cI857 repressor. For this the DNA cassette which is preferably located on a vector is transformed into a bacterial cell which contains a gene coding for a temperature-sensitive cI repressor. This gene may also be present on a vector (Remaut et al., Gene 15 (1981), 81-93). On the other hand it is possible to use a bacterial cell which contains such a repressor gene in its chromosome e.g. E. coli M5219 (cf. e.g. Shimatake and Rosenberg, Nature 292 (1981), 128).

Mutants which are resistant to lysis at different temperatures can be identified in a simple manner by culturing the bacterial cells transformed with a lysis cassette which contain the mutated operator DNA

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sequences. Up to now it has been possible to identify several mutants which are resistant to a lysis at temperatures up to 33°C, 35°C, 37°C and 39°C. These bacteria contain mutated operator DNA sequences which allow binding of the repressor up to the respective temperature. A particularly preferred example is a mutant to which the cI857 repressor binds up to a temperature of about 37°C. Compared to the wild-type the mutation is a single base substitution in the O_R2 section of the lambda O_R operator region. The sequence of this mutated lambda O_R operator is shown in SEQ ID NO.2.

An additional subject matter of the invention are mutated O_R or O_L operator sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding of a repressor and which are obtainable by the selection methods described above. The mutated O_R or O_L operator sequences preferably have an increased thermostability with regard to the binding of a temperature-sensitive repressor and in particular of the temperature-sensitive cI repressor. The mutated operator sequences particularly preferably have a thermostability that is increased by about 3 to 10°C, in particular by about 7 to 9°C compared to the wild-type sequence.

Since the selection method according to the invention is preferably carried out on O_R or O_L operator sequences which are derived from the phage lambda, the present invention in particular concerns mutated lambda O_R or O_L operator sequences which are variants of the O_R operator sequences shown in SEQ ID NO.1 or variants of the O_L operator sequences shown in SEQ ID NO.3. Variant in this connection is understood as an operator sequence which differs from the wild-type sequence in at least one

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sequence position by insertion, deletion or substitution of bases. The differences are particularly preferably in the region of the sections O_R1, O_R2 or O_R3 and O_L1, O_L2 and O_L3. A specific example of a mutated lambda operator sequence according to the invention is the lambda=O_R operator sequence shown in SEQ ID NO.2.

The mutated operator sequences allow the production of new temperature-regulated systems for gene expression in which microorganisms and in particular bacteria can be cultured in a repressed state at variable temperatures and preferably at higher temperatures than have been previously possible in particular at 33 to 39°C. Hence a subject matter of the invention is the use of the mutated O_R or O_L operator sequences for the temperature-regulated expression of genes in bacteria and in particular in gram-negative bacteria such as E. coli. Combination of a wild-type O_R or O_L operator region and at least one operator region which contains a mutated operator sequence according to the invention or combination of several operator regions which contain mutated operator sequences according to the invention with different thermostabilities even enables a temperature-regulated sequential expression of genes.

Vectors and bacterial strains in which the inventive mutated operator sequences can be used for the temperature-regulated expression of genes are familiar to a person skilled in the art. In this case the expression systems known from the prior art containing the lambda cI857 repressor in combination with a suitable promoter e.g. the lambda P_L or lambda P_R promoter can be used (cf. e.g. Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd Edition, 1989, Cold Spring Harbor Laboratory Press, New York,

17.11-17.12).

A further subject matter of the present invention is a nucleic acid comprising a bacterial expression control sequence i.e. a sequence containing a promoter and operator regions which contains a mutated O_R or O_L operator sequence according to the invention in operative linkage with a protein-coding sequence. The protein-coding sequence can for example be a sequence coding for a eukaryotic protein or polypeptide or a bacterial gene e.g. the E-lysis gene.

An additional subject matter of the present invention is a vector which contains at least one copy of the bacterial expression control sequence in operative linkage with the protein-coding sequence. This vector can be any prokaryotic vector e.g. a chromosomal vector such as a bacteriophage or an extrachromosomal vector such as a plasmid. Suitable prokaryotic vectors are described for example by Sambrook et al., Supra, chapters 1-4.

Yet a further subject matter of the present invention is a bacterial cell which is transformed with a nucleic acid according to the invention or with a vector according to the invention. In a preferred embodiment the cell is a gram-negative prokaryotic cell, particularly preferably an E. coli cell. The cell preferably contains the nucleic acid or the vector integrated into its chromosome and in addition contains a gene for a cI repressor from a lambdoid phage in particular the gene for the lambda cI857 repressor.

A particularly preferred application of the mutated

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operators according to the invention is in the field of vaccine production. So-called "bacterial ghosts" are known as vaccines from the prior art i.e. bacterial coats that can be prepared from gram-negative bacteria such as *E. coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Actinobacillus pleuropneumoniae* etc. by means of protein-E-induced lysis. These ghosts whose cell surface properties and repertoire of surface antigens that can be recognized by the immune system are very similar to the active pathogen, produce a protective cellular or/and humoral immune response in various animal models.

The process for preparing the ghosts is based on the stringent controlled expression of the E-lysis gene from PhiX174 whose expression product forms a tunnel through the bacterial cell wall coat and thus leads to a pouring out of the cell contents of the host cell. This lethal gene for the cells can be regulated by means of a lambda repressor e.g. the temperature-sensitive lambda repressor cI857 which, as described above, loses its function at temperatures above 30°C. As a result, the bacterial cultures that have previously been used to produce bacterial ghosts have had to be cultured at low temperatures, preferably at 28°C.

Although this method leads to satisfactory results with regard to the immunogenicity of the ghosts that are produced, an improvement of the bacterial culture is urgently required since the repertoire of antigenic determinants on the bacterial surface can change depending on the external conditions. Since pathogenic bacteria which infect humans or animals usually colonize at an environmental temperature of 37 to 39°C, this natural environmental temperature should also be

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maintained during the production process for the ghosts.

A process for producing bacterial ghosts which achieves this object is provided by using the mutated operator sequences according to the invention. These operator sequences allow growth of the bacteria up to a temperature range of preferably 35 to 39°C and allow lysis when the temperature is increased from 37 to 42°C. This changed lysis behaviour enables the pathogens to be cultured near to the body temperature of the vaccine candidate which is extremely important for the composition of the external membrane. Furthermore the new lysis cassette can also be used as a safety cassette in live vaccines since for example in humans the inoculated bacteria are killed when fever is induced (39°C).

Hence a subject matter of the invention is a vaccine composition which contains a live bacterial cell according to the invention as the active ingredient optionally together with pharmaceutically tolerated auxiliary substances, additives and carrier substances. The live bacterial cell contains a nucleic acid comprising a bacterial expression control sequence with a mutated operator sequence in operative linkage preferably with a lysis gene. Yet a further subject matter of the present invention is a vaccine composition which contains a bacterial ghost as an active ingredient optionally together with pharmaceutically tolerated auxiliary substances, additives and carrier substances wherein the bacterial ghost is obtainable by culturing a bacterial cell according to the invention at temperatures of 35 - 39°C and subsequently lysing the bacterial cell by increasing the temperature. Bacterial cells suitable as vaccines are in particular gram-

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negative bacteria such as E. coli for example the strains STEC, EHEC, O78:K80, Salmonellae such as S.choleraesuis, S.enteritidis and S.typhimurium, Pasteurella multocida, Pasteurella haemolytica, Bordetella bronchiseptica, Klebsiella pneumoniae, Actinobacillus pleuropneumoniae, Haemophilus influenzae, Vibrio cholerae, Helicobacter pylori, Alcaligenes eutrophus, Campylobacter jejuni and Pseudomonas aeruginosa.

The vaccine compositions modified according to the invention can be transferred orally, aerogenically or parenterally to the vaccine candidates. The route which the corresponding microorganisms naturally select for the infection and for the initial stages of establishing an infectious disease are preferably selected for the application of the vaccine. Since all surface properties are retained in the vaccines according to the invention, this application can result in a local induction of the immune response as also occurs in the natural infection process.

As described above the use of mutated operator sequences according to the invention enables the development of vaccines that can be lysed in a controlled manner when a target temperature is exceeded. Furthermore it is, however, also possible to provide a cold-sensitive suicide cassette which on release into the environment kills gram-negative bacteria that are used as a live vaccine. Hence combination of two genetic regulation systems enables the bacteria to die as a result of the expression of a suicide gene when a target value for the environmental temperature is exceeded. This safety cassette ensures that the live vaccines are killed even if they are eliminated from the organisms.

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Hence the invention concerns a nucleic acid comprising (a) a first bacterial expression control sequence which contains an O_R or O_L operator sequence from a lambdoid phage and to which a first temperature-sensitive cI repressor from lambdoid phages can bind in operative linkage with a sequence coding for a second repressor wherein the second repressor cannot bind to the first bacterial expression sequence and (b) a second bacterial expression control sequence to which the second repressor can bind which is in operative linkage with a suicide gene.

The components (a) and (b) can be covalently linked together e.g. be present on a single vector or be separate from one another e.g. present on different vectors or be located separately or together on the chromosome of a recipient bacterium.

Yet a further subject matter of the present invention is a bacterial cell which contains at least one copy of a nucleic acid as described above. In addition the bacterial cell advantageously contains a gene for the first repressor. The first repressor is preferably the temperature-sensitive cI857 repressor.

The safety cassette according to the invention preferably contains a gene which codes for a temperature-sensitive cI repressor e.g. the repressor cI857 and a gene which codes for a second repressor wherein this gene is under the control of a lambda promoter/operator region to which the temperature-sensitive repressor binds. The second repressor in turn controls the expression of another gene e.g. a suicide gene such as the E-lysis gene. The temperature-sensitive

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lambda repressor is inactive at 37°C so that the second repressor is expressed which in turn represses the expression of the suicide gene.

When the temperature is reduced the temperature-sensitive lambda repressor binds to the operator so that the expression of the second repressor is blocked which leads to an expression of the suicide gene. A first expression control sequence is preferred for this safety cassette which contains the mutated lambda operator since this enables an improved and more rapid activation of the suicide gene.

The second repressor can be any repressor e.g. a lac repressor. However, it is preferable to use an additional repressor from lambdoid phages e.g. cI from the phage 434 which is not temperature-sensitive and binds to its own operator sequence but does not bind to the sequence recognized by the lambda repressor cI857.

It is particularly preferable for the development of live vaccines to incorporate a heat as well as a cold regulation element. This incorporation is preferably achieved by homologous recombination into the chromosome of the vaccine bacterium.

Thus the present invention also concerns a bacterial cell which, in addition to the two components (a) and (b), contains a third bacterial expression control sequence as component (c) which contains a mutated operator sequence according to the invention in operative linkage with a suicide gene.

These bacterial cells can also be used in vaccine compositions and especially for live vaccines. In this manner it is possible to produce heat or/and cold-sensitive safe live vaccines which lead to death of the vaccine bacteria when the body temperature of the vaccine candidate is increased e.g. by fever or/and when they are excreted into the environment.

It is intended to additionally elucidate the invention by the following figures, sequence protocols and examples.

- Fig. 1a shows a schematic representation of a lysis cassette of the prior art comprising a lambda O_R wild-type region, the lambda cI857 gene under the control of the promoter P_{RM} and the E lysis gene under the control of the promoter P_R ;
- Fig. 1b shows a schematic representation of a lysis cassette according to the invention which contains a mutated lambda O_R sequence;
- Fig. 2a shows a schematic representation of a cold-sensitive safety cassette comprising a wild-type (pCS1) or mutated (pCSJ1) O_R operator sequence, the lambda-cI857 gene under the control of the promoter P_{RM} , the gene of the lacI repressor under the control of P_R and the E-lysis gene under the control of the lac promoter/operator system at a temperature at which the temperature-sensitive lambda repressor cI857 does not bind to the lambda O_R sequence;
- Fig. 2b shows a schematic representation of the safety cassette according to Fig. 2a at a temperature at which the lambda repressor cI857 binds to the lambda O_R operator;

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- Fig. 3 shows the lysis curve of bacterial cells (optical density versus time) which contain a plasmid with the lysis cassette shown in Fig. 1b;
- Fig. 4 shows the lysis curve of a bacterial cell which contains a cold-sensitive safety cassette with the wild-type O_R operator and
- Fig. 5 shows a comparison of lysis curves of bacterial cells which contain a cold-sensitive safety lysis cassette with the wild-type O_R operator (pSC1) or the mutated operator (pCSJ1);
- Fig. 6a shows a schematic representation of a cold-sensitive safety cassette comprising a wild-type (pCS2) or mutated (pCSJ2) O_R operator sequence, the lambda cI857 gene under the control of the promoter P_{RM} , the gene of the phage 434 cI repressor under the control of lambda P_R and the E lysis gene under the control of the 434 O_R ($P_{RM}-P_R$) promoter/operator system at a temperature at which the temperature-sensitive lambda repressor cI857 does not bind to the lambda O_R sequence,
- Fig. 6b shows a schematic representation of the safety cassette according to Fig. 6a at a temperature at which the lambda repressor cI857 binds to the lambda O_R operator;

SEQ ID NO.1 shows the nucleotide sequence of the lambda O_R operator; the operator sequence O_{R3} extends from position 11 - 27; the operator sequence O_{R2} extends from position 34 - 41; the operator sequence O_{R1} extends from position 58 - 74;

SEQ ID NO.2 shows the nucleotide sequence of a mutated lambda O_R operator which, compared to the wild-type sequence, has a substitution of

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T → C at position 42;

SEQ ID NO.3 shows the nucleotide sequence of the lambda O_L operator; the operator sequence O_{L3} extends from position 11 - 27; the operator sequence O_{L2} extends from position 31 - 47; the operator sequence O_{L1} extends from position 55 - 70:

SEQ ID NO. 4 to 6

show a 1601 bp long DNA fragment of the plasmid pAW12; bp 1 - 983 are derived from the bacteriophage lambda (position 37125 - 38107; cf. Sanger et al., J.Mol.Biol. 162 (1982), 729-773) and contain the lambda cI857 gene (position 816-106; SEQ ID NO.5) as well as the mutated O_R operator region (mutation at position 858 T → C); bp 1023 - 1601 are derived from the phage PhiX174 (position 447 - 1026; cf. Sanger et al., J.Mol.Biol. 125 (1978), 225-246) and contain the E-lysis gene (position 1144 - 1416; SEQ ID NO.6);

SEQ ID NO. 7 to 10

shows a 2834 bp long DNA fragment of the plasmid pCSJ; bp 1 - 983 are derived from the bacteriophage lambda (position 37125 - 38107) and contain the cI857 gene (position 816 - 106; SEQ ID NO.5) as well as the mutated lambda O_R region (mutation at position 858 T → C; bp 990 - 2230 are derived from the E. coli lac operon subcloned on the plasmid pMC7 (Calos, Nature 274 (1978), 762-765) and contain the lacI repressor gene (bp 1025 - 2104; SEQ ID NO.9) and the lac promoter/operator; bp 2256 - 2834 are derived from the bacteriophage

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PhiX174 (position 447 - 1026) and contain the E-lysis gene (bp 2377 - 2649; SEQ ID NO.10).

Examples

Example 1:

1.1 Random mutagenesis of the lambda O_R operator region

The plasmid pAW12 (Witte and Lubitz, Eur.J.Biochem. 180 (1989), 393-398) was selected as the starting material which contains the lysis gene E from the bacteriophage PhiX174 under the control of the lambda P_R promoter as well as the associated repressor gene cI857. The aim of this experiment was to change the lysis cassette so that the lysis gene E is not already activated at 30°C but at higher temperatures. For this the E. coli mutator strain ES1578 (Wu et al., (1990), supra) was transformed with the lysis plasmid and a selection was carried out for clones with a changed temperature profile of cell lysis.

For this the mutated clones produced by the transformation were detected after being stamped onto test plates containing lysis selective medium (LB containing 1 % SDS) and incubated at different temperatures (e.g. 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C). The changed lysis profile of the lysis cassette in liquid culture was exactly determined by plasmid extraction and subsequent transformation into a non-mutator test strain.

The type of mutation was determined by subcloning the

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mutagenized lysis cassettes into a sequencing plasmid. In addition the lysis gene E was substituted by the β -galactosidase gene for a functional check. It was then possible on the basis of a simple β -gal test to quantitatively measure the repressed or active state of the gene cassette.

In this manner it was possible to obtain several clones with a different temperature lysis profile. These clones allowed growth of the bacteria in a temperature range of 33-39°C and did not lead to lysis of the bacteria until the temperature was further increased to 37-42°C.

A mutation of the O_R operator region (SEQ ID NO.2) was identified by sequencing a mutated clone which had a thermostability up to 37°C.

1.2 Verification of the mutation

In order to verify the mutation obtained in example 1.1. a site-specific mutagenesis of the lambda O_R wild-type sequence was carried out using an oligonucleotide.

The mutagenesis was carried out according to the protocol of Kunkel (Proc.Natl.Acad.Sci. USA 82 (1985), 488-492).

4 ml overnight culture of the E. coli strain CJ236 (dut^- , ung^-) was added to 50 ml LB medium (+ 10 μ g/ml chloramphenicol and 0.25 μ g/ml uridine) and shaken for 30 min at 37°C. Then 100 μ l M13 phages was added and it was incubated for 6 h at 37°C.

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The culture was centrifuged in 2 SS34 centrifuge tubes for 10 min at 14000 rpm and 4°C, the supernatant was decanted into new tubes and again centrifuged for further purification.

The phages were precipitated for 1 h at 4°C by addition of 5 ml 5 x polyethylene glycol/NaCl. They were then centrifuged for 10 min at 14000 rpm and 4°C and the supernatant was discarded.

The pellet was dried, suspended in 0.8 ml TES buffer (0.1 M Tris HCl, pH 8; 0.3 M NaCl; 1 mM EDTA) and incubated for 1 h at 4°C. The suspension was divided into 2 Eppendorf vessels and centrifuged for 5 min at 5000 rpm. The supernatant in which the disrupted phages were located was removed and subjected to a phenol/chloroform extraction to isolate the DNA. The resulting DNA was precipitated with a 2.5-fold volume of 96 % ethanol, washed with 70 % ethanol and taken up in 60 µl H₂O.

An oligonucleotide with the sequence 5'-GTA AAA TAG TCA ACA CGC GCG GTG TTA GAT ATT TAT C-3' was phosphorylated. For this 20 µl H₂O, 20 µl oligonucleotide (20 ng), 4.5 µl kinase buffer (Stratagene) and 0.5 µl polynucleotide kinase (5 U, Stratagene) was incubated for 1 h at 37°C. Then 7 µl 0.1 M EDTA was added and it was heated for 10 min to 65°C.

For the annealing 20 µl phosphorylated oligonucleotide, 3.5 µl single-stranded DNA template (1 µg ssDNA produced as described above) and 1.4 µl 20 x SSC buffer were heated for 5 min to 70°C, slowly cooled to 25°C and then

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placed on ice.

For the extension 10 μ l of the reaction mixture from the annealing mixture, 37.5 μ l XL buffer (27 mM Hepes pH 7.8, 5 mM of each dNTP, 13 mM MgCl₂, 2.7 mM dithiothreitol, 1.3 mM ATP, 1 μ l ligase (1 U, Boehringer Mannheim), 1.5 μ l T4 polymerase (1.5 U, Boehringer Mannheim), 1.5 μ l T4 gene32 protein (8 μ g, Boehringer Mannheim) were incubated for 10 min on ice, 10 min at room temperature and 2 h at 37°C. After 1 h 1 μ l ligase and 1 μ l T4 DNA polymerase was added. After completion of the incubation the reaction was stopped by adding 3 μ l 0.25 M EDTA.

For the transformation 100 μ l competent E. coli cells JM103 (Messing et al., Nucleic Acids Res. 9 (1981), 309-321) was admixed with 10 μ l DNA from the extension mixture and incubated for 1 h or more on ice. After a heat shock for 2.5 min at 42°C, 0.2 ml fresh JM103 cells was added in the logarithmic growth phase. The cells were mixed with 3 ml soft agar and inoculated on an LB agar plate. They were subsequently incubated overnight at 37°C.

In order to identify the mutants, plaques were pricked out with a Pasteur pipette and used to inoculate 5 ml LB medium to which 400 μ l of an overnight culture of E. coli JM103 had been added. After 3 h growth at 37°C, the cells were centrifuged. Double-stranded M13 plasmids were obtained from the cell pellet by means of plasmid preparation. Single-stranded M13 phages could be isolated from the supernatant.

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Example 2:

Analysis of the mutagenized lysis cassettes

Figures 1 and 2 shows different E-specific lysis cassettes with different temperature inductions of the lysis function.

In Fig. 1a which contains the wild-type lambda O_R operator sequence, the function of the E-lysis gene is repressed up to 30°C by the cI857-coded repressor protein on the preceding lambda P_R promoter/operator region. cI857-specific repressor molecules are thermally inactivated at temperatures above 30°C and the expression of the E gene is induced. Fig. 1b shows the plasmid pAWJ12 which contains a mutated operator sequence (SEQ ID NO.2) so that the repression of the lysis function of the gene E by cI857 occurs up to 37°C and the lysis function is not induced until 39°C or higher temperatures are reached.

The function of a cold-sensitive safety cassette is elucidated in Fig. 2. Fig. 2a shows that the formation of lacI-specific repressor molecules which in turn repress the expression of the E gene is induced in the plasmids pCS1 (wild-type operator) and pCSJ1 (mutated operator) at a temperature of $\geq 32^\circ\text{C}$ (pCS1) or $\geq 39^\circ\text{C}$ (pCSJ1). At a temperature below 30°C (pCS1) or 37°C (pCSJ1) functional cI857 repressor molecules are formed which suppress the formation of lacI-specific repressor molecules and thus allow the expression of the E gene (Fig. 2b). In the plasmid pCSJ1 the concomitant cell lysis occurs more rapidly than in pCS1.

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Fig. 3 shows the lysis curve of a bacterial cell containing the plasmid pAWJ12 (mutated operator). 3 hours after beginning the culture, the temperature was maintained at 37°C in an aliquot of the bacterial cells and increased in two other aliquots to 38 and 42°C. At 37°C there was a further growth of the bacteria whereas a lysis already occurred at 38°C. The lysis is considerably increased at 42°C.

Figures 4 and 5 show the function of a cold-sensitive safety cassette. In Fig. 4 bacterial cells which contained the plasmid pCS1 (wild-type operator) were subjected to a temperature change from 37 to 28°C. This reduction in temperature led to the E-lysis gene being switched off and cell death (decrease of the optical density).

Fig. 5 shows a comparison of the lysis rate of bacteria which contain the plasmid pCS1 (wild-type operator) and the plasmid pCSJ1 (mutated operator). It can be seen that the lysis occurs much more rapidly in the bacteria which contain the mutated operator.

Fig. 6 shows a further cold-sensitive safety cassette. At temperatures at which the lambda cI857 repressor does not bind to the operator the plasmids pCS2 (wild-type operator) and pCSJ2 (mutated operator) form cI-434 repressor molecules which repress the expression of the E gene (Fig. 6a). Formation of cI-434-specific repressor molecules is prevented thus allowing expression of the E gene at a temperature at which the cI857 repressor binds to the lambda operator (Fig. 6b).

Example 3:In vivo analysis of cold-sensitive lysis cassettes

The killing of bacteria by lowering the temperature after passage through a mouse intestine and excretion into the faeces was determined.

For this 10^{10} E. coli bacteria were administered once to Balb/c mice and the excreted number of bacteria in the faeces was determined. The evaluation was carried out on E.coli-specific Endo plates (Endo, "Zentralbl. Bakt. I Orig." 35 (1904) 109-110) using tetracyclin as a marker for the plasmids used. The incubation was carried out at 28°C.

Results:

In the experimental groups E. coli NM522 (pCS2), E. coli MC4100 (pCS1) and E. coli MC4100 (pCSJ1) there was a reduction in the germ count compared to an E. coli NM522 (pAWJ-lac) control of at least 99.9 %, 98 % and 80 % measured 10 h and 20 h after administering the E. coli bacteria.

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SEQUENCE PROTOCOL

(1) GENERAL INFORMATION:

→ (i) APPLICANT:

- (A) NAME: Prof.DR. Werner Lubitz
- (B) ROAD: Schoenborngasse 12/7
- (C) CITY: Vienna
- (E) COUNTRY: Austria
- (F) POSTAL CODE: 1080

(ii) TITLE OF INVENTION: New systems for the regulation
of gene expression

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER-READABLE FORM:

- (A) DATA CARRIER: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0,
Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 82 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(vi) INITIAL ORIGIN:

- (A) ORGANISM: lambda OR operator (wild-type)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ACGTTAAATC TATCACCGCA AGGGATAAAT ATCTAACACC GTGCGTGTG ACTATTTAC	60
CTCTGGCGGT GATAATGGTT GC	82

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 82 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

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(vi) INITIAL ORIGIN:

(A) ORGANISM: lambda OR operator (mutant)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ACGTTAAATC TATCACCGCA AGGGATAAAAT ATCTAACACC GCGCGTGTG ACTATTAC	60
CTCTGGCGGT GATAATGGTT GC	82

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 85 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(vi) INITIAL ORIGIN:

(A) ORGANISM: lambda OL operator (wild-type)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ACATACAGAT AACCATCTGC GGTGATAAAAT TATCTCTGGC GGTGTTGACA TAAATACCAC	60
TGGCGGTGAT ACTGAGCACA TCAGC	85

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1601 base pairs
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: double strand
- (D) TOPOLOGY: both

(vi) INITIAL ORIGIN:

(A) ORGANISM: pAW12 fragment

(xi) CHARACTERISTICS:

- (A) NAME/KEY: CDS
- (B) LOCATION: complement (106..816)

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(ix) CHARACTERISTICS:

- 5 (A) NAME/KEY: CDS
 (B) LOCATION: 1144..1416

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

10

15	ATTTACTATG TTATGTTCTG AGGGGAGTGA AAATTCCCTT AATTGATGA AGATTCTTGC	60
15	TCAATTGTTA TCAGCTATGC GCCGACCAGA ACACCTTGCAC GATCAGCCAA ACGTCTCTTC	120
	AGGCCACTGA CTAGCGATAA CTTTCCCCAC AACGGAACAA CTCTCATTGC ATGGGATCAT	180
20	TGGGTACTGT GGGTTTAGTG GTTGTAAAAA CACCTGACCG CTATCCCTGA TCAGTTCTT	240
	GAAGGTAAAC TCATCACCCC CAAGTCTGGC TATGCAGAAA TCACCTGGCT CAACAGCCTG	300
	CTCAGGGTCA ACGAGAAATTA ACATTCCGTC AGGAAAGCTT GGCTTGGAGC CTGTTGGTGC	360
25	GGTCATGGAA TTACCTTAA CCTCAAGCCA GAATGCAGAA TCACCTGGCTT TTTTGGTTGT	420
	GCTTACCCAT CTCTCCGCAT CACCTTTGGT AAAGGTTCTA AGCTTAGGTG AGAACATCCC	480
30	TGCCTGAACA TGAGAAAAAA CAGGGTACTC ATACTCACTT CTAAGTGACG GCTGCATACT	540
	AACCGCTTCA TACATCTCGT AGATTTCTCT GGCGATTGAA GGGCTAAATT CTTCAACGCT	600
	AACTTTGAGA ATTTTTGTAA GCAATGCGC GTTATAAGCA TTTAATGCAT TGATGCCATT	660
35	AAATAAAGCA CCAACGCCCTG ACTGCCCAT CCCCATCTTG TCTGCGACAG ATTCCCTGGGA	720
	TAAGCCAAGT TCATTTTCT TTTTTTCATA AATTGCTTTA AGGCGACGTG CGTCCTCAAG	780
40	CTGCTCTTGT GTTAATGGTT TCTTTTTGT GTCATACGT TAAATCTATC ACCGCAAGGG	840
	ATAAAATATCT AACACCGCGC GTGTTGACTA TTTTACCTCT GGCGGTGATA ATGGTTGCAT	900
	GTACTAAGTA GGTGTATGG AACAAACGCAT AACCTGAAA GATTATGCAA TGCGCTTGG	960
45	GCAAACCAAG ACAGCTAAAG ATCCTCTAGA GTCGACCTGC AGGCATGCAA GCTTATCGAA	1020
	TTCTCATTCA GGCTTCTGCC GTTTGGATT TAACCGAAGA TGATTTCGAT TTTCTGACGA	1080
50	GTAACAAAGT TTGGATTGCT ACTGACCGCT CTCGTGCTCG TCGCTGCGTT GAGGCTTGC	1140
	TTT ATG GTA CGC TGG ACT TTG TGG GAT ACC CTC GCT TTC CTG CTC CTG	1188
	Met Val Arg Trp Thr Leu Trp Asp Thr Leu Ala Phe Leu Leu Leu	
	1 5 10 15	
55	TTG AGT TTA TTG CTG CCG TCA TTG CTT ATT ATG TTC ATC CCG TCA ACA	1236
	Leu Ser Leu Leu Pro Ser Leu Leu Ile Met Phe Ile Pro Ser Thr	
	20 25 30	
60	TTC AAA CGG CCT GTC TCA TCA TGG AAG GCG CTG AAT TTA CGG AAA ACA	1284
	Phe Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu Arg Lys Thr	
	35 40 45	

65

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TTA TCA ATG GCG TCG AGC GTC CGG TTA AAG CCG CTG AAT TGT TCG CGT Leu Leu Met Ala Ser Ser Val Arg Leu Lys Pro Leu Asn Cys Ser Arg 50 55 60	1332
5 TTA CCT TGC GTG TAC GCG CAG GAA ACA CTG ACG TTC TTA CTG ACG CAG Leu Pro Cys Val Tyr Ala Gln Glu Thr Leu Thr Phe Leu Leu Thr Gln 65 70 75	1380
10 AAG AAA ACG TGC GTC AAA AAT TAC GTG CAG AAG GAG TGATGTAATG Lys Lys Thr Cys Val Lys Asn Tyr Val Gln Lys Glu 80 85 90	1426
15 TCTAAAGGTA AAAAACGTTTC TGGCGCTCGC CCTGGTCGTC CGCAGCCGTT GCGAGGTTACT	1486
20 15 AAAGGCAAGC GTAAAGGCAG TCGTCTTGG TATGTAGGTG GTCAACAATT TTAATTGCAG GGGCTTCGGC CCTTACTTGA GGATAAAATTA TGTCTAATAT TCAAACTGGC GCCGA	1546
	1601

20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
 25 (A) LENGTH: 237 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: protein

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

35 Met Ser Thr Lys Lys Pro Leu Thr Gln Glu Gln Leu Glu Asp Ala 1 5 10 15
Arg Arg Leu Lys Ala Ile Tyr Glu Lys Lys Lys Asn Glu Leu Gly Leu 20 25 30
40 40 Ser Gln Glu Ser Val Ala Asp Lys Met Gly Met Gly Gln Ser Gly Val 35 40 45
Gly Ala Leu Phe Asn Gly Ile Asn Ala Leu Asn Ala Tyr Asn Ala Ala 45 50 55 60
Leu Leu Thr Lys Ile Leu Lys Val Ser Val Glu Glu Phe Ser Pro Ser 65 70 75 80
50 50 Ile Ala Arg Glu Ile Tyr Glu Met Tyr Glu Ala Val Ser Met Gln Pro 85 90 95
Ser Leu Arg Ser Glu Tyr Glu Tyr Pro Val Phe Ser His Val Gln Ala 100 105 110
55 55 Gly Met Phe Ser Pro Lys Leu Arg Thr Phe Thr Lys Gly Asp Ala Glu 115 120 125
60 60 Arg Trp Val Ser Thr Thr Lys Lys Ala Ser Asp Ser Ala Phe Trp Leu 130 135 140
Glu Val Glu Gly Asn Ser Met Thr Ala Pro Thr Gly Ser Lys Pro Ser 145 150 155 160

- 28 -

Phe Pro Asp Gly Met Leu Ile Leu Val Asp Pro Glu Gln Ala Val Glu
 165 170 175
 Pro Gly Asp Phe Cys Ile Ala Arg Leu Gly Gly Asp Glu Phe Thr Phe
 5 180 185 190
 Lys Lys Leu Ile Arg Asp Ser Gly Gln Val Phe Leu Gln Pro Leu Asn
 195 200 205
 10 Pro Gln Tyr Pro Met Ile Pro Cys Asn Glu Ser Cys Ser Val Val Gly
 210 215 220
 Lys Val Ile Ala Ser Gln Trp Pro Glu Glu Thr Phe Gly
 225 230 235
 15

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 91 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) TYPE OF MOLECULE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

30
 Met Val Arg Trp Thr Leu Trp Asp Thr Leu Ala Phe Leu Leu Leu
 1 5 10 15
 35 Ser Leu Leu Leu Pro Ser Leu Leu Ile Met Phe Ile Pro Ser Thr Phe
 20 25 30
 Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu Arg Lys Thr Leu
 35 40 45
 40 Leu Met Ala Ser Ser Val Arg Leu Lys Pro Leu Asn Cys Ser Arg Leu
 50 55 60
 Pro Cys Val Tyr Ala Gln Glu Thr Leu Thr Phe Leu Leu Thr Gln Lys
 45 65 70 75 80
 Lys Thr Cys Val Lys Asn Tyr Val Gln Lys Glu
 85 90

50
 (2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2834 base pairs
 - (B) TYPE: nucleotide
 - (C) STRANDEDNESS: double strand
 - (D) TOPOLOGY: both

60

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(vi) INITIAL ORIGIN:

(A) ORGANISM: pCSJ fragment

5 (xi) CHARACTERISTICS:

(A) NAME/KEY: CDS

(B) LOCATION: complement (106..816)

— (ix) CHARACTERISTICS:

(A) NAME/KEY: CDS

(B) LOCATION: 1025..2104

(ix) CHARACTERISTICS:

(A) NAME/KEY: CDS

(B) LOCATION: 2377..2649

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

20

	ATTTACTATG TTATGTTCTG AGGGGAGTGA AAATTCCCCT AATTCGATGA AGATTCTTGC	60	
25	TCAATTGTTA TCAGCTATGC GCGGACCAAGA ACACCTTGCC GATCAGCCAA ACGTCTCTTC	120	
	AGGCCACTGA CTAGCGATAA CTTTCCCCAC AACGGAACAA CTCTCATTCG ATGGGATCAT	180	
30	TGGGTACTGT GGGTTTAGTG GTTGTAAAAA CACCTGACCG CTATCCCTGA TCAGTTCTT	240	
	GAAGGTAAAC TCATCACCCCC CAAGTCTGGC TATGCCAGAAA TCACCTGGCT CAAACAGCCTG	300	
	CTCAGGGTCA ACGAGAATTAA ACATTCCGTC AGGAAAGCTT GGCTTGAGC CTGTTGGTGC	360	
35	GGTCATGGAA TTACCTTCAA CCTCAAGCCA GAATGCAGAA TCACTGGCTT TTTTGGTTGT	420	
	GCTTACCCAT CTCTCCGCAT CACCTTTGGT AAAGGTTCTA AGCTTAGGTG AGAACATCCC	480	
40	TGCCTGAACA TGAGAAAAAA CAGGGTACTC ATACTCACTT CTAAGTGACG GCTGCATACT	540	
	AACCGCTTCA TACATCTCGT AGATTCTCT GGCGATTGAA GGGCTAAATT CTTCAACGCT	600	
	AACTTTGAGA ATTTTTGTAA GCAATGCGGC GTTATAAGCA TTTAATGCAT TGATGCCATT	660	
45	AAATAAAGCA CCAACGCCTG ACTGCCCAT CCCCATCTTG TCTGCGACAG ATTCCGGGA	720	
	TAAGCCAAGT TCATTTTCT TTTTTCTATA AATTGCTTTA AGGCGACGTG CGTCCTCAAG	780	
50	CTGCTCTTGT GTTAATGGTT TCTTTTTGT GCTCATACGT TAAATCTATC ACCGCAAGGG	840	
	ATAAAATATCT AACACCGCGC GTGTTGACTA TTTTACCTCT GGCGGTGATA ATGGTTGCAT	900	
	GTACTAAGTA GGTTGTATGG AACAAACGCAT AACCCCTGAAA GATTATGCAA TGGCTTTGG	960	
55	GCAAACCAAG ACAGCTAAAG ATCCTCTAGA GCGCCCGGAA GAGAGTCAAT TCAGGGTGGT	1020	
	GAAT GTG AAA CCA GTA ACG TTA TAC GAT GTC GCA GAG TAT GCC GGT GTC	1069	
	Val Lys Pro Val Thr Leu Tyr Asp Val Ala Glu Tyr Ala Gly Val		
	95	100	105
60	TCT TAT CAG ACC GTT TCC CGC GTG GTG AAC CAG GCG AGC CAC GTT TCT	1117	
	Ser Tyr Gln Thr Val Ser Arg Val Val Asn Gln Ala Ser His Val Ser		
	110	115	120

- 30 -

125	GCG AAA ACG CGG GAA AAA GTG GAA GCG GCG ATG GCG GAG CTG AAT TAC Ala Lys Thr Arg Glu Lys Val Glu Ala Ala Met Ala Glu Leu Asn Tyr 125 130 135	1165
5	ATT CCC AAC CGC GTG GCA CAA CAA CTG GCG GGC AAA CAG TCG TTG CTG Ile Pro Asn Arg Val Ala Gln Gln Leu Ala Gly Lys Gln Ser Leu Leu 140 145 150	1213
10	ATT GGC GTT GCC ACC TCC AGT CTG GCC CTG CAC GCG CCG TCG CAA ATT Ile Gly Val Ala Thr Ser Ser Leu Ala Leu His Ala Pro Ser Gln Ile 155 160 165 170	1261
15	GTC GCG GCG ATT AAA TCT CGC GCC GAT CAA CTG GGT GCC AGC GTG GTG Val Ala Ala Ile Lys Ser Arg Ala Asp Gln Leu Gly Ala Ser Val Val 175 180 185	1309
20	GTG TCG ATG GTA GAA CGA AGC GGC GTC GAA GCC TGT AAA GCG GCG GTG Val Ser Met Val Glu Arg Ser Gly Val Glu Ala Cys Lys Ala Ala Val 190 195 200	1357
25	CAC AAT CTT CTC GCG CAA CGC GTC AGT GGG CTG ATC ATT AAC TAT CCG His Asn Leu Leu Ala Gln Arg Val Ser Gly Leu Ile Ile Asn Tyr Pro 205 210 215	1405
30	CTG GAT GAC CAG GAT GCC ATT GCT GTG GAA GCT GCC TGC ACT AAT GTT Leu Asp Asp Gln Asp Ala Ile Ala Val Glu Ala Ala Cys Thr Asn Val 220 225 230	1453
35	CCG GCG TTA TTT CTT GAT GTC TCT GAC CAG ACA CCC ATC AAC AGT ATT Pro Ala Leu Phe Leu Asp Val Ser Asp Gln Thr Pro Ile Asn Ser Ile 235 240 245 250	1501
40	ATT TTC TCC CAT GAA GAC GGT ACG CGA CTG GGC GTG GAG CAT CTG GTC Ile Phe Ser His Glu Asp Gly Thr Arg Leu Gly Val Glu His Leu Val 255 260 265	1549
45	GCA TTG GGT CAC CAG CAA ATC GCG CTG TTA GCG GGC CCA TTA AGT TCT Ala Leu Gly His Gln Gln Ile Ala Leu Leu Ala Gly Pro Leu Ser Ser 270 275 280	1597
50	GTC TCG GCG CGT CTG CGT CTG GCT GGC TGG CAT AAA TAT CTC ACT CGC Val Ser Ala Arg Leu Arg Leu Ala Gly Trp His Lys Tyr Leu Thr Arg 285 290 295	1645
55	AAT CAA ATT CAG CCG ATA GCG GAA CGG GAA GGC GAC TGG AGT GCC ATG Asn Gln Ile Gln Pro Ile Ala Glu Arg Glu Gly Asp Trp Ser Ala Met 300 305 310	1693
60	TCC GGT TTT CAA CAA ACC ATG CAA ATG CTG AAT GAG GGC ATC GTT CCC Ser Gly Phe Gln Gln Thr Met Gln Met Leu Asn Glu Gly Ile Val Pro 315 320 325 330	1741
65	ACT GCG ATG CTG GTT GCC AAC GAT CAG ATG GCG CTG GGC GCA ATG CGC Thr Ala Met Leu Val Ala Asn Asp Gln Met Ala Leu Gly Ala Met Arg 335 340 345	1789
70	GCC ATT ACC GAG TCC GGG CTG CGC GTT GGT GCG GAT ATC TCG GTA GTG Ala Ile Thr Glu Ser Gly Leu Arg Val Gly Ala Asp Ile Ser Val Val 350 355 360	1837
75	GGA TAC GAC GAT ACC GAA GAC AGC TCA TGT TAT ATC CCG CCG TCA ACC Gly Tyr Asp Asp Thr Glu Asp Ser Ser Cys Tyr Ile Pro Pro Ser Thr 365 370 375	1885
80	ACC ATC AAA CAG GAT TTT CGC CTG CTG GGG CAA ACC AGC GTG GAC CGC Thr Ile Lys Gln Asp Phe Arg Leu Leu Gly Gln Thr Ser Val Asp Arg 380 385 390	1933

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TTG CTG CAA CTC TCT CAG GGC CAG GCG GTG AAG GGC AAT CAG CTG TTG Leu Leu Gln Leu Ser Gln Gly Gln Ala Val Lys Gly Asn Gln Leu Leu 395 400 405 410	1981
5 CCC GTC TCA CTG GTG AAA AGA AAA ACC ACC CTG GCG CCC AAT ACG CAA Pro Val Ser Leu Val Lys Arg Lys Thr Thr Leu Ala Pro Asn Thr Gln 415 420 425	2029
10 ACC GCC TCT CCC CGC GCG TTG GCC GAT TCA TTA ATG CAG CTG GCA CGA Thr Ala Ser Pro Arg Ala Leu Ala Asp Ser Leu Met Gln Leu Ala Arg 430 435 440	2077
15 CAG GTT TCC CGA CTG GAA ACC GGG CAG TGAGCGCAAC GCAATTAAATG Gln Val Ser Arg Leu Glu Ser Gly Gln 445 450	2124
20 TGAGTTAGCT CACTCATTAG GCACCCCCAGG CTTTACACTT TATGCTTCCG GCTCGTATGT	2184
25 TGTGTGGAAT TGTGAGCGGA TAACAATTTC ACACAGGAAA CAGCTCTGCA GGCATGCAAG CTTATCGAAC TCTCATTCAAG GCTTCTGCCG TTTTGGATTT AACCGAAGAT GATTTCGATT TTCTGACGAG TAACAAAGTT TGGATTGCTA CTGACCGCTC TCGTGCTCGT CGCTGCGTTG	2244 2304 2364
30 25 AGGCTTGCGT TT ATG GTA CGC TGG ACT TTG TGG GAT ACC CTC GCT TTC Met Val Arg Trp Thr Leu Trp Asp Thr Leu Ala Phe 1 5 10	2412
35 CTG CTC CTG TTG AGT TTA TTG CTG CCG TCA TTG CTT ATT ATG TTC ATC Leu Leu Leu Ser Leu Leu Pro Ser Leu Leu Ile Met Phe Ile 15 20 25	2460
40 35 CCG TCA ACA TTC AAA CGG CCT GTC TCA TCA TGG AAG GCG CTG AAT TTA Pro Ser Thr Phe Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu 30 35 40	2508
45 CCG AAA ACA TTA TTA ATG GCG TCG AGC GTC CCG TTA AAG CCG CTG AAT Arg Lys Thr Leu Leu Met Ala Ser Ser Val Arg Leu Lys Pro Leu Asn 45 50 55 60	2556
50 55 TGT TCG CGT TTA CCT TGC GTG TAC GCG CAG GAA ACA CTG ACG TTC TTA Cys Ser Arg Leu Pro Cys Val Tyr Ala Gln Glu Thr Leu Thr Phe Leu 65 70 75	2604
55 60 65 CTG ACG CAG AAG AAA ACG TGC GTC AAA AAT TAC GTG CAG AAG GAG Leu Thr Gln Lys Lys Thr Cys Val Lys Asn Tyr Val Gln Lys Glu 80 85 90	2649
70 75 80 TGATGTAATG TCTAAAGGTA AAAAACGTTG TGGCGCTCGC CCTGGTCGTC CGCAGCCGTT	2709
80 85 90 85 GCGAGGTACT AAAGGCAAGC GTAAAGGCAG TCGTCTTGG TATGTAGGTG GTCAACAATT	2769
90 95 TTAATTGCAG GGGCTTCGGC CCTTACTTGA GGATAAATTA TGTCTAATAT TCAGACTGGC	2829
100 105 110 115 120 125 130 135 140 145 150 155 160 165 170 175 180 185 190 195 200 205 210 215 220 225 230 235 240 245 250 255 260 265 270 275 280 285 290 295 300 305 310 315 320 325 330 335 340 345 350 355 360 365 370 375 380 385 390 395 400 405 410 415 420 425 430 435 440 445 450 455 460 465 470 475 480 485 490 495 500 505 510 515 520 525 530 535 540 545 550 555 560 565 570 575 580 585 590 595 600 605 610 615 620 625 630 635 640 645 650 655 660 665 670 675 680 685 690 695 700 705 710 715 720 725 730 735 740 745 750 755 760 765 770 775 780 785 790 795 800 805 810 815 820 825 830 835 840 845 850 855 860 865 870 875 880 885 890 895 900 905 910 915 920 925 930 935 940 945 950 955 960 965 970 975 980 985 990 995 1000 1005 1010 1015 1020 1025 1030 1035 1040 1045 1050 1055 1060 1065 1070 1075 1080 1085 1090 1095 1100 1105 1110 1115 1120 1125 1130 1135 1140 1145 1150 1155 1160 1165 1170 1175 1180 1185 1190 1195 1200 1205 1210 1215 1220 1225 1230 1235 1240 1245 1250 1255 1260 1265 1270 1275 1280 1285 1290 1295 1300 1305 1310 1315 1320 1325 1330 1335 1340 1345 1350 1355 1360 1365 1370 1375 1380 1385 1390 1395 1400 1405 1410 1415 1420 1425 1430 1435 1440 1445 1450 1455 1460 1465 1470 1475 1480 1485 1490 1495 1500 1505 1510 1515 1520 1525 1530 1535 1540 1545 1550 1555 1560 1565 1570 1575 1580 1585 1590 1595 1600 1605 1610 1615 1620 1625 1630 1635 1640 1645 1650 1655 1660 1665 1670 1675 1680 1685 1690 1695 1700 1705 1710 1715 1720 1725 1730 1735 1740 1745 1750 1755 1760 1765 1770 1775 1780 1785 1790 1795 1800 1805 1810 1815 1820 1825 1830 1835 1840 1845 1850 1855 1860 1865 1870 1875 1880 1885 1890 1895 1900 1905 1910 1915 1920 1925 1930 1935 1940 1945 1950 1955 1960 1965 1970 1975 1980 1985 1990 1995 2000 2005 2010 2015 2020 2025 2030 2035 2040 2045 2050 2055 2060 2065 2070 2075 2080 2085 2090 2095 2100 2105 2110 2115 2120 2125 2130 2135 2140 2145 2150 2155 2160 2165 2170 2175 2180 2185 2190 2195 2200 2205 2210 2215 2220 2225 2230 2235 2240 2245 2250 2255 2260 2265 2270 2275 2280 2285 2290 2295 2300 2305 2310 2315 2320 2325 2330 2335 2340 2345 2350 2355 2360 2365 2370 2375 2380 2385 2390 2395 2400 2405 2410 2415 2420 2425 2430 2435 2440 2445 2450 2455 2460 2465 2470 2475 2480 2485 2490 2495 2500 2505 2510 2515 2520 2525 2530 2535 2540 2545 2550 2555 2560 2565 2570 2575 2580 2585 2590 2595 2600 2605 2610 2615 2620 2625 2630 2635 2640 2645 2650 2655 2660 2665 2670 2675 2680 2685 2690 2695 2700 2705 2710 2715 2720 2725 2730 2735 2740 2745 2750 2755 2760 2765 2770 2775 2780 2785 2790 2795 2800 2805 2810 2815 2820 2825 2830 2835 2840 2845 2850 2855 2860 2865 2870 2875 2880 2885 2890 2895 2900 2905 2910 2915 2920 2925 2930 2935 2940 2945 2950 2955 2960 2965 2970 2975 2980 2985 2990 2995 3000 3005 3010 3015 3020 3025 3030 3035 3040 3045 3050 3055 3060 3065 3070 3075 3080 3085 3090 3095 3100 3105 3110 3115 3120 3125 3130 3135 3140 3145 3150 3155 3160 3165 3170 3175 3180 3185 3190 3195 3200 3205 3210 3215 3220 3225 3230 3235 3240 3245 3250 3255 3260 3265 3270 3275 3280 3285 3290 3295 3300 3305 3310 3315 3320 3325 3330 3335 3340 3345 3350 3355 3360 3365 3370 3375 3380 3385 3390 3395 3400 3405 3410 3415 3420 3425 3430 3435 3440 3445 3450 3455 3460 3465 3470 3475 3480 3485 3490 3495 3500 3505 3510 3515 3520 3525 3530 3535 3540 3545 3550 3555 3560 3565 3570 3575 3580 3585 3590 3595 3600 3605 3610 3615 3620 3625 3630 3635 3640 3645 3650 3655 3660 3665 3670 3675 3680 3685 3690 3695 3700 3705 3710 3715 3720 3725 3730 3735 3740 3745 3750 3755 3760 3765 3770 3775 3780 3785 3790 3795 3800 3805 3810 3815 3820 3825 3830 3835 3840 3845 3850 3855 3860 3865 3870 3875 3880 3885 3890 3895 3900 3905 3910 3915 3920 3925 3930 3935 3940 3945 3950 3955 3960 3965 3970 3975 3980 3985 3990 3995 4000 4005 4010 4015 4020 4025 4030 4035 4040 4045 4050 4055 4060 4065 4070 4075 4080 4085 4090 4095 4100 4105 4110 4115 4120 4125 4130 4135 4140 4145 4150 4155 4160 4165 4170 4175 4180 4185 4190 4195 4200 4205 4210 4215 4220 4225 4230 4235 4240 4245 4250 4255 4260 4265 4270 4275 4280 4285 4290 4295 4300 4305 4310 4315 4320 4325 4330 4335 4340 4345 4350 4355 4360 4365 4370 4375 4380 4385 4390 4395 4400 4405 4410 4415 4420 4425 4430 4435 4440 4445 4450 4455 4460 4465 4470 4475 4480 4485 4490 4495 4500 4505 4510 4515 4520 4525 4530 4535 4540 4545 4550 4555 4560 4565 4570 4575 4580 4585 4590 4595 4600 4605 4610 4615 4620 4625 4630 4635 4640 4645 4650 4655 4660 4665 4670 4675 4680 4685 4690 4695 4700 4705 4710 4715 4720 4725 4730 4735 4740 4745 4750 4755 4760 4765 4770 4775 4780 4785 4790 4795 4800 4805 4810 4815 4820 4825 4830 4835 4840 4845 4850 4855 4860 4865 4870 4875 4880 4885 4890 4895 4900 4905 4910 4915 4920 4925 4930 4935 4940 4945 4950 4955 4960 4965 4970 4975 4980 4985 4990 4995 5000 5005 5010 5015 5020 5025 5030 5035 5040 5045 5050 5055 5060 5065 5070 5075 5080 5085 5090 5095 5100 5105 5110 5115 5120 5125 5130 5135 5140 5145 5150 5155 5160 5165 5170 5175 5180 5185 5190 5195 5200 5205 5210 5215 5220 5225 5230 5235 5240 5245 5250 5255 5260 5265 5270 5275 5280 5285 5290 5295 5300 5305 5310 5315 5320 5325 5330 5335 5340 5345 5350 5355 5360 5365 5370 5375 5380 5385 5390 5395 5400 5405 5410 5415 5420 5425 5430 5435 5440 5445 5450 5455 5460 5465 5470 5475 5480 5485 5490 5495 5500 5505 5510 5515 5520 5525 5530 5535 5540 5545 5550 5555 5560 5565 5570 5575 5580 5585 5590 5595 5600 5605 5610 5615 5620 5625 5630 5635 5640 5645 5650 5655 5660 5665 5670 5675 5680 5685 5690 5695 5700 5705 5710 5715 5720 5725 5730 5735 5740 5745 5750 5755 5760 5765 5770 5775 5780 5785 5790 5795 5800 5805 5810 5815 5820 5825 5830 5835 5840 5845 5850 5855 5860 5865 5870 5875 5880 5885 5890 5895 5900 5905 5910 5915 5920 5925 5930 5935 5940 5945 5950 5955 5960 5965 5970 5975 5980 5985 5990 5995 6000 6005 6010 6015 6020 6025 6030 6035 6040 6045 6050 6055 6060 6065 6070 6075 6080 6085 6090 6095 6100 6105 6110 6115 6120 6125 6130 6135 6140 6145 6150 6155 6160 6165 6170 6175 6180 6185 6190 6195 6200 6205 6210 6215 6220 6225 6230 6235 6240 6245 6250 6255 6260 6265 6270 6275 6280 6285 6290 6295 6300 6305 6310 6315 6320 6325 6330 6335 6340 6345 6350 6355 6360 6365 6370 6375 6380 6385 6390 6395 6400 6405 6410 6415 6420 6425 6430 6435 6440 6445 6450 6455 6460 6465 6470 6475 6480 6485 6490 6495 6500 6505 6510 6515 6520 6525 6530 6535 6540 6545 6550 6555 6560 6565 6570 6575 6580 6585 6590 6595 6600 6605 6610 6615 6620 6625 6630 6635 6640 6645 6650 6655 6660 6665 6670 6675 6680 6685 6690 6695 6700 6705 6710 6715 6720 6725 6730 6735 6740 6745 6750 6755 6760 6765 6770 6775 6780 6785 6790 6795 6800 6805 6810 6815 6820 6825 6830 6835 6840 6845 6850 6855 6860 6865 6870 6875 6880 6885 6890 6895 6900 6905 6910 6915 6920 6925 6930 6935 6940 6945 6950 6955 6960 6965 6970 6975 6980 6985 6990 6995 7000 7005 7010 7015 7020 7025 7030 7035 7040 7045 7050 7055 7060 7065 7070 7075 7080 7085 7090 7095 7100 7105 7110 7115 7120 7125 7130 7135 7140 7145 7150 7155 7160 7165 7170 7175 7180 7185 7190 7195 7200 7205 7210 7215 7220 7225 7230 7235 7240 7245 7250 7255 7260 7265 7270 7275 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9280 9285 9290 9295 9300 9305 9310 9315 9320 9325 9330 9335 9340 9345 9350 9355 9360 9365 9370 9375 9380 9385 9390 9395 9400 9405 9410 9415 9420 9425 9430 9435 9440 9445 9450 9455 9460 9465 9470 9475 9480 9485 9490 9495 9500 9505 9510 9515 9520 9525 9530 9535 9540 9545 9550 9555 9560 9565 9570 9575 9580 9585 9590 9595 9600 9605 9610 9615 9620 9625 9630 9635 9640 9645 9650 9655 9660 9665 9670 9675 9680 9685 9690 9695 9700 9705 9710 9715 9720 9725 9730 9735 9740 9745 9750 9755 9760 9765 9770 9775 9780 9785 9790 9795 9800 9805 9810 9815 9820 9825 9830 9835 9840 9845 9850 9855 9860 9865 9870 9875 9880 9885 9890 9895 9900 9905 9910 9915 9920 9925 9930 9935 9940 9945 9950 9955 9960 9965 9970 9975 9980 9985 9990 9995 10000 10005 10010 10015 10020 10025 10030 10035 10040 1004	

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(ii) TYPE OF MOLECULE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

5	Met Ser Thr Lys Lys Lys Pro Leu Thr Gln Glu Gln Leu Glu Asp Ala
	1 5 10 15
	Arg Arg Leu Lys Ala Ile Tyr Glu Lys Lys Asn Glu Leu Gly Leu
	20 25 30
10	Ser Gln Glu Ser Val Ala Asp Lys Met Gly Met Gly Gln Ser Gly Val
	35 40 45
15	Gly Ala Leu Phe Asn Gly Ile Asn Ala Leu Asn Ala Tyr Asn Ala Ala
	50 55 60
	Leu Leu Thr Lys Ile Leu Lys Val Ser Val Glu Glu Phe Ser Pro Ser
	65 70 75 80
20	Ile Ala Arg Glu Ile Tyr Glu Met Tyr Glu Ala Val Ser Met Gln Pro
	85 90 95
	Ser Leu Arg Ser Glu Tyr Glu Tyr Pro Val Phe Ser His Val Gln Ala
	100 105 110
25	Gly Met Phe Ser Pro Lys Leu Arg Thr Phe Thr Lys Gly Asp Ala Glu
	115 120 125
30	Arg Trp Val Ser Thr Thr Lys Lys Ala Ser Asp Ser Ala Phe Trp Leu
	130 135 140
	Glu Val Glu Gly Asn Ser Met Thr Ala Pro Thr Gly Ser Lys Pro Ser
	145 150 155 160
35	Phe Pro Asp Gly Met Leu Ile Leu Val Asp Pro Glu Gln Ala Val Glu
	165 170 175
	Pro Gly Asp Phe Cys Ile Ala Arg Leu Gly Gly Asp Glu Phe Thr Phe
	180 185 190
40	Lys Lys Leu Ile Arg Asp Ser Gly Gln Val Phe Leu Gln Pro Leu Asn
	195 200 205
45	Pro Gln Tyr Pro Met Ile Pro Cys Asn Glu Ser Cys Ser Val Val Gly
	210 215 220
	Lys Val Ile Ala Ser Gln Trp Pro Glu Glu Thr Phe Gly
	225 230 235
50	

(2) INFORMATION FOR SEQ ID NO: 9:

- | | |
|----|--------------------------------|
| 55 | (i) SEQUENCE CHARACTERISTICS: |
| | (A) LENGTH: 360 amino acids |
| | (B) TYPE: amino acid |
| | (D) TOPOLOGY: linear |
| 60 | (ii) TYPE OF MOLECULE: protein |

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

	Val Lys Pro Val Thr Leu Tyr Asp Val Ala Glu Tyr Ala Gly Val Ser	
5	1 5 10 15	
	Tyr Gln Thr Val Ser Arg Val Val Asn Gln Ala Ser His Val Ser Ala	
	20 25 30	
10	Lys Thr Arg Glu Lys Val Glu Ala Ala Met Ala Glu Leu Asn Tyr Ile	
	35 40 45	
	Pro Asn Arg Val Ala Gln Gln Leu Ala Gly Lys Gln Ser Leu Leu Ile	
	50 55 60	
15	Gly Val Ala Thr Ser Ser Leu Ala Leu His Ala Pro Ser Gln Ile Val	
	65 70 75 80	
	Ala Ala Ile Lys Ser Arg Ala Asp Gln Leu Gly Ala Ser Val Val Val	
20	85 90 95	
	Ser Met Val Glu Arg Ser Gly Val Glu Ala Cys Lys Ala Ala Val His	
	100 105 110	
25	Asn Leu Leu Ala Gln Arg Val Ser Gly Leu Ile Ile Asn Tyr Pro Leu	
	115 120 125	
	Asp Asp Gln Asp Ala Ile Ala Val Glu Ala Ala Cys Thr Asn Val Pro	
	130 135 140	
30	Ala Leu Phe Leu Asp Val Ser Asp Gln Thr Pro Ile Asn Ser Ile Ile	
	145 150 155 160	
	Phe Ser His Glu Asp Gly Thr Arg Leu Gly Val Glu His Leu Val Ala	
35	165 170 175	
	Leu Gly His Gln Gln Ile Ala Leu Leu Ala Gly Pro Leu Ser Ser Val	
	180 185 190	
40	Ser Ala Arg Leu Arg Leu Ala Gly Trp His Lys Tyr Leu Thr Arg Asn	
	195 200 205	
	Gln Ile Gln Pro Ile Ala Glu Arg Glu Gly Asp Trp Ser Ala Met Ser	
	210 215 220	
45	Gly Phe Gln Gln Thr Met Gln Met Leu Asn Glu Gly Ile Val Pro Thr	
	225 230 235 240	
	Ala Met Leu Val Ala Asn Asp Gln Met Ala Leu Gly Ala Met Arg Ala	
50	245 250 255	
	Ile Thr Glu Ser Gly Leu Arg Val Gly Ala Asp Ile Ser Val Val Gly	
	260 265 270	
55	Tyr Asp Asp Thr Glu Asp Ser Ser Cys Tyr Ile Pro Pro Ser Thr Thr	
	275 280 285	
	Ile Lys Gln Asp Phe Arg Leu Leu Gly Gln Thr Ser Val Asp Arg Leu	
	290 295 300	
60	Leu Gln Leu Ser Gln Gly Gln Ala Val Lys Gly Asn Gln Leu Leu Pro	
	305 310 315 320	
	Val Ser Leu Val Lys Arg Lys Thr Thr Leu Ala Pro Asn Thr Gln Thr	
65	325 330 335	

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Ala Ser Pro Arg Ala Leu Ala Asp Ser Leu Met Gln Leu Ala Arg Gln
340 345 350

5 Val Ser Arg Leu Glu Ser Gly Gln
355 360

10 (2)- INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 91 amino acids
(B) TYPE: amino acid
15 (D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

20

Met Val Arg Trp Thr Leu Trp Asp Thr Leu Ala Phe Leu Leu Leu
1 5 10 15

25 Ser Leu Leu Leu Pro Ser Leu Leu Ile Met Phe Ile Pro Ser Thr Phe
20 25 30

30 Lys Arg Pro Val Ser Ser Trp Lys Ala Leu Asn Leu Arg Lys Thr Leu
35 40 45

Leu Met Ala Ser Ser Val Arg Leu Lys Pro Leu Asn Cys Ser Arg Leu
50 55 60

35 Pro Cys Val Tyr Ala Gln Glu Thr Leu Thr Phe Leu Leu Thr Gln Lys
65 70 75 80

40 Lys Thr Cys Val Lys Asn Tyr Val Gln Lys Glu
85 90

Claims

1. Method for selecting mutated O_R or O_L operator DNA sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding a repressor,
wherein
 - (a) a DNA cassette is prepared which contains a selection gene under the operative control of an expression control sequence comprising at least one O_R or O_L operator sequence from a lambdoid phage and a promoter,
 - (b) the operator DNA sequence is subjected to a mutagenesis and
 - (c) the mutated operator DNA sequences are analysed.
2. Method as claimed in claim 1,
wherein
the lambdoid phages are selected from the group comprising the phage lambda, phage 21, phage 22, phage 82, phage 424, phage 434, phage D326, phage DLP12, phage gamma, phage HK022, phage P4, phage Phi80, phage Phi81, coliphage 186 and recombinant variants thereof.
3. Method as claimed in claim 2,
wherein
the phage lambda or recombinant variants thereof are used.

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4. Method as claimed in claim 3,
wherein
an operator DNA sequence from the operator regions O_R or/and O_L of the phage lambda is used.
5. Method as claimed in one of the claims 1 - 4,
wherein
the E-lysis gene from the phage PhiX174 is used as the selection gene.
6. Method as claimed in one of the claims 1 - 5,
wherein
the operator DNA sequence is subjected to a site-specific mutagenesis by oligonucleotides or a selection is carried out in a mutator bacterial strain.
7. Method as claimed in one of the claims 1 - 6,
wherein
the mutated operator DNA sequences are analysed by determining their ability to bind to a temperature-sensitive cI repressor.
8. Method as claimed in claim 7,
wherein
the temperature-sensitive lambda repressor cI857 is used.
9. Mutated O_R or O_L operator sequences from lambdoid phages which have a different thermostability compared to the wild-type sequence with regard to binding of a repressor and are obtainable by a method as claimed in one of the claims 1 - 8.

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10. Mutated O_R or O_L operator sequences from lambdoid phages which have an increased thermostability compared to the wild-type sequence with regard to binding of a temperature-sensitive repressor and
are obtainable by a method as claimed in one of the claims 1 - 8.
11. Mutated O_R or O_L operator sequence as claimed in claim 10,
wherein
it has an approximately 3 - 10°C increased thermostability.
12. Mutated O_R or O_L operator sequence as claimed in claim 10,
wherein
it has an approximately 7 - 9°C increased thermostability.
13. Mutated lambda O_R or O_L operator sequence as claimed in one of the claims 9 - 12, which is a variant of the sequences shown in SEQ ID NO.1 or SEQ ID NO.3.
14. Mutated lambda O_R operator sequence comprising the sequence shown in SEQ ID NO.2.
15. Use of a mutated O_R or O_L operator sequence as claimed in one of the claims 9 - 14 for the temperature-regulated expression of genes in bacterial cells.

16. Use of a combination of (a) a wild-type O_R or O_L operator region and at least one operator region which contains a mutated O_R or O_L operator sequence as claimed in one of the claims 9 - 14 or (b)
several operator regions which contain mutated O_R or O_L operator sequences as claimed in one of the claims 9 - 14 with different thermostabilities for the temperature-regulated sequential expression of genes.
17. Use as claimed in claim 15 or 16,
wherein
the bacterial cells contain a gene for a cI repressor from lambdoid phages for the regulation of gene expression.
18. Use as claimed in claim 17,
wherein
the bacterial cells contain the gene for the lambda cI857 repressor.
19. Nucleic acid comprising a bacterial expression control sequence which contains a mutated O_R or O_L operator sequence as claimed in one of the claims 9 - 14 in operative linkage with a protein-coding sequence.
20. Nucleic acid as claimed in claim 19,
wherein
the protein-coding sequence is a suicide gene.
21. Nucleic acid as claimed in claim 20,
wherein
the expression control sequence contains a lambda P_L or P_R promoter.

22. Vector,
wherein
it contains at least one copy of a nucleic acid as
claimed in one of the claims 19 - 21.
23. Vector as claimed in claim 22,
wherein
it is a bacterial chromosomal vector.
24. Vector as claimed in claim 22,
wherein
it is a bacterial extrachromosomal plasmid.
25. Bacterial cell,
wherein
it is transformed with a nucleic acid as claimed in
one of the claims 19 - 21 or with a vector as
claimed in one of the claims 22 - 24.
26. Bacterial cell as claimed in claim 25,
wherein
it contains the nucleic acid or the vector
integrated into its chromosome.
27. Bacterial cell as claimed in claim 25 or 26,
wherein
it additionally contains a gene for a cI repressor
from lambdoid phages.
28. Bacterial cell as claimed in claim 27,
wherein
it contains the gene for the lambda cI857
repressor.

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29. Vaccine composition,
wherein
it contains a live bacterial cell as claimed in one
of the claims 26 - 28 as an active ingredient
optionally with pharmaceutically acceptable
auxiliary substances, additives and carrier
substances.
30. Vaccine composition,
wherein
it contains a bacterial ghost as the active
ingredient optionally with pharmaceutically
acceptable auxiliary substances, additives and
carrier substances in which the bacterial ghost can
be obtained by culturing a bacterial cell as
claimed in one of the claims 25 - 28 at
temperatures of 35 - 39°C and subsequently lysing
the bacterial cell by increasing the temperature.
31. Nucleic acid comprising (a) a first bacterial
expression control sequence which contains an O_R or
O_L operator sequence from a lambdoid phage and to
which a first cI repressor from lambdoid phages can
bind, in operative linkage with a sequence coding
for a second repressor wherein the second repressor
cannot bind to the first bacterial expression
sequence and (b) a second bacterial expression
control sequence to which the second repressor can
bind in operative linkage with a suicide gene.
32. Bacterial cell,
wherein
it contains at least one copy of a nucleic acid as
claimed in claim 31.

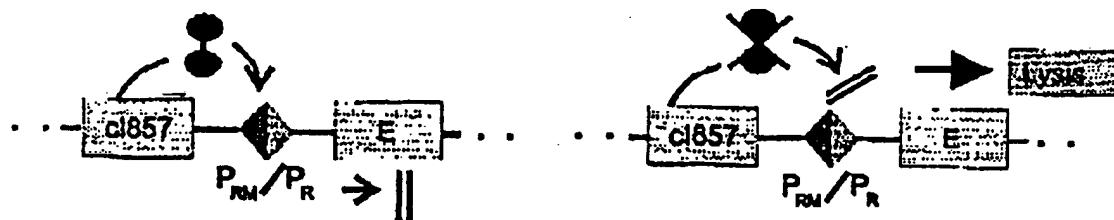
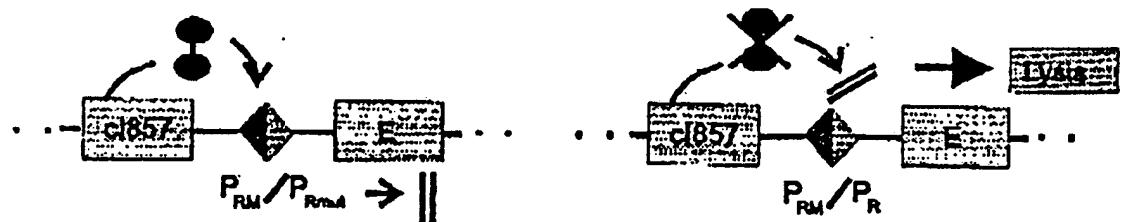
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33. Bacterial cell as claimed in claim 32,
wherein
it additionally contains a gene for the first
repressor.
34. Bacterial cell as claimed in claim 32 or 33,
wherein
it contains the first bacterial expression control
sequence of a mutated operator sequence as claimed
in one of the claims 9 - 14.
35. Bacterial cell as claimed in one of the claims 32 -
34 additionally comprising (c) a third bacterial
expression control sequence which contains a
mutated operator sequence as claimed in one of the
claims 9 - 14 in operative linkage with a suicide
gene.
36. Vaccine composition,
wherein
it contains a live bacterial cell as claimed in one
of the claims 32 - 35 as the active ingredient
optionally together with pharmaceutically
acceptable auxiliary substances, additives and
carrier substances.
37. Use of vaccine compositions as claimed in claim 29
or 36 as heat-sensitive or/and cold-sensitive safe
live vaccines.

PCTWELTORGANISATION FÜR GEISTIGES EIGENTUM
Internationales BüroINTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE
INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

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<p>(21) Internationales Aktenzeichen: PCI/EP97/04560</p> <p>(22) Internationales Anmeldedatum: 21. August 1997 (21.08.97)</p> <p>(30) Prioritätsdaten: 196 33 698:8 21. August 1996 (21.08.96) DE</p> <p>(71)(72) Anmelder und Erfinder: LUBITZ, Werner [AT/AT]; Schönborngasse 12/7, A-1080 Wien (AT).</p> <p>(72) Erfinder; und</p> <p>(75) Erfinder/Anmelder (nur für US): JECHLINGER, Wolfgang [AT/AT]; Strozzigasse 38/12, A-1080 Wien (AT). SZOSTAK, Michael [AT/AT]; In den Schnäbeln 9/3, A-2344 Maria Enzersdorf (AT).</p> <p>(74) Anwälte: WEICKMANN, H. usw.; Kopernikusstrasse 9, D-81679 München (DE).</p>		<p>(81) Bestimmungsstaaten: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO Patent (GH, KE, LS, MW, SD, SZ, UG, ZW), curasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Veröffentlicht <i>Mit internationalem Recherchenbericht. Vor Ablauf der für Änderungen der Ansprüche zugelassenen Frist. Veröffentlichung wird wiederholt falls Änderungen eintreffen.</i></p> <p>(88) Veröffentlichungsdatum des internationalen Recherchenberichts: 26. März 1998 (26.03.98)</p>	
<p>(54) Title: NEW SYSTEMS FOR REGULATING GENETIC EXPRESSION</p> <p>(54) Bezeichnung: NEUE SYSTEME ZUR REGULATION DER GENEXPRESION</p> <p>(57) Abstract</p> <p>The present invention concerns a process for selecting new PR- or PL-operator sequences of lambdoid phages which, compared to wild-type sequences, have a different thermostability for the binding of a repressor. In addition, the invention discloses new mutated PR- or PL- operator sequences and their use for temperature-regulated expression of genes and for producing improved vaccines.</p> <p>(57) Zusammenfassung</p> <p>Die vorliegende Erfindung betrifft ein Verfahren zur Selektion neuer PR- oder PL-Operatorsequenzen aus lambdoiden Phagen, die eine im Vergleich zur Wildtypsequenz unterschiedliche Thermostabilität hinsichtlich der Bindung eines Repressors aufweisen. Weiterhin werden neue mutierte PR- oder PL-Operatorsequenzen sowie deren Verwendung zur temperaturregulierten Expression von Genen und zur Herstellung besserer Impfstoffe offenbart.</p>			

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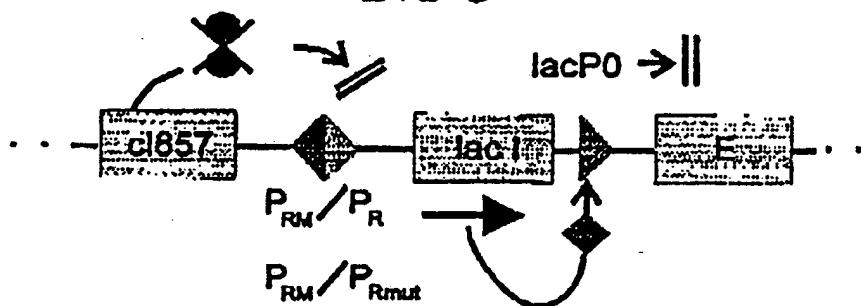
1a) pAW12 $\leq 30^\circ\text{C}$ $\geq 30^\circ\text{C}$ b) pAWJ12 $\leq 37^\circ\text{C}$ $\geq 39^\circ\text{C}$ 

2a)

 $\geq 32^\circ\text{C}$

pCS1

pCSJ1

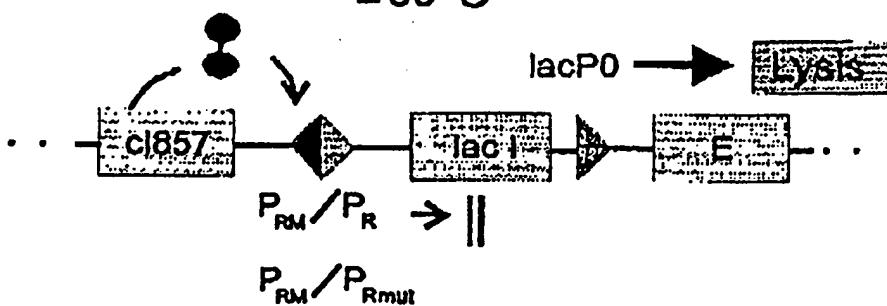


2b)

 $\leq 30^\circ\text{C}$

pCS1

pCSJ1



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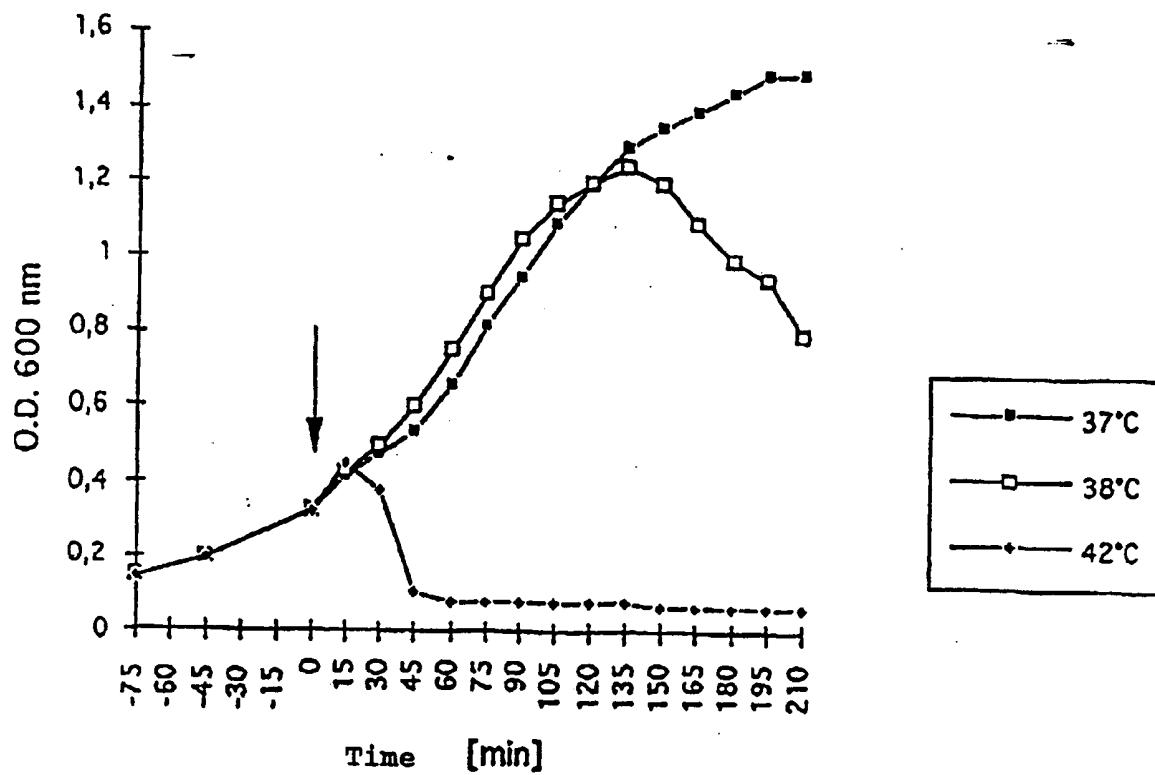


Fig. 3: Growth of *E. coli* NM522 (pAWJ12) when the temperature is changed from 28°C to higher temperatures (↓)

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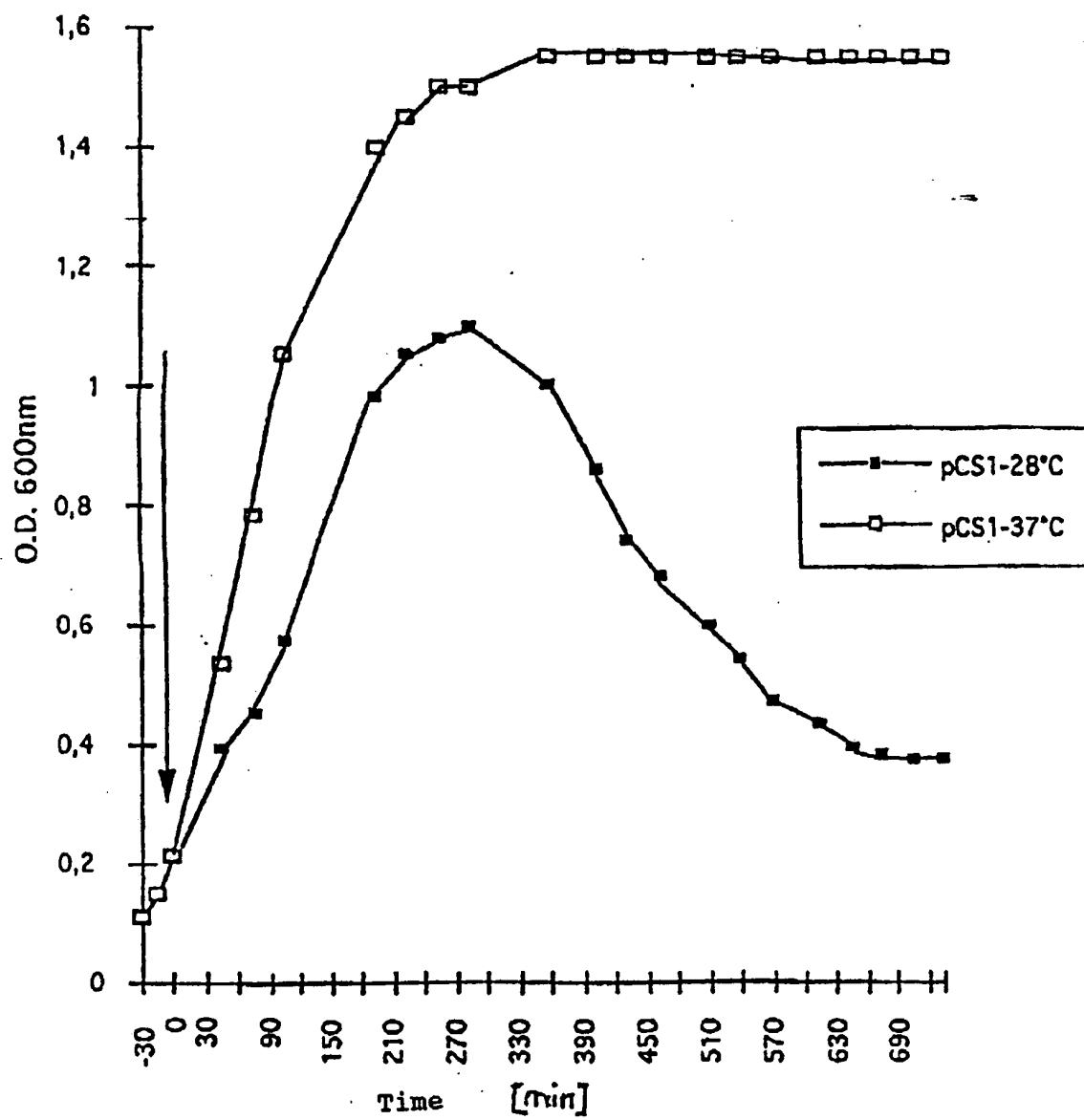


Fig. 4: Growth of *E. coli* MC4100 (pCS1) when the temperature is changed from 37°C to 28°C (↓)

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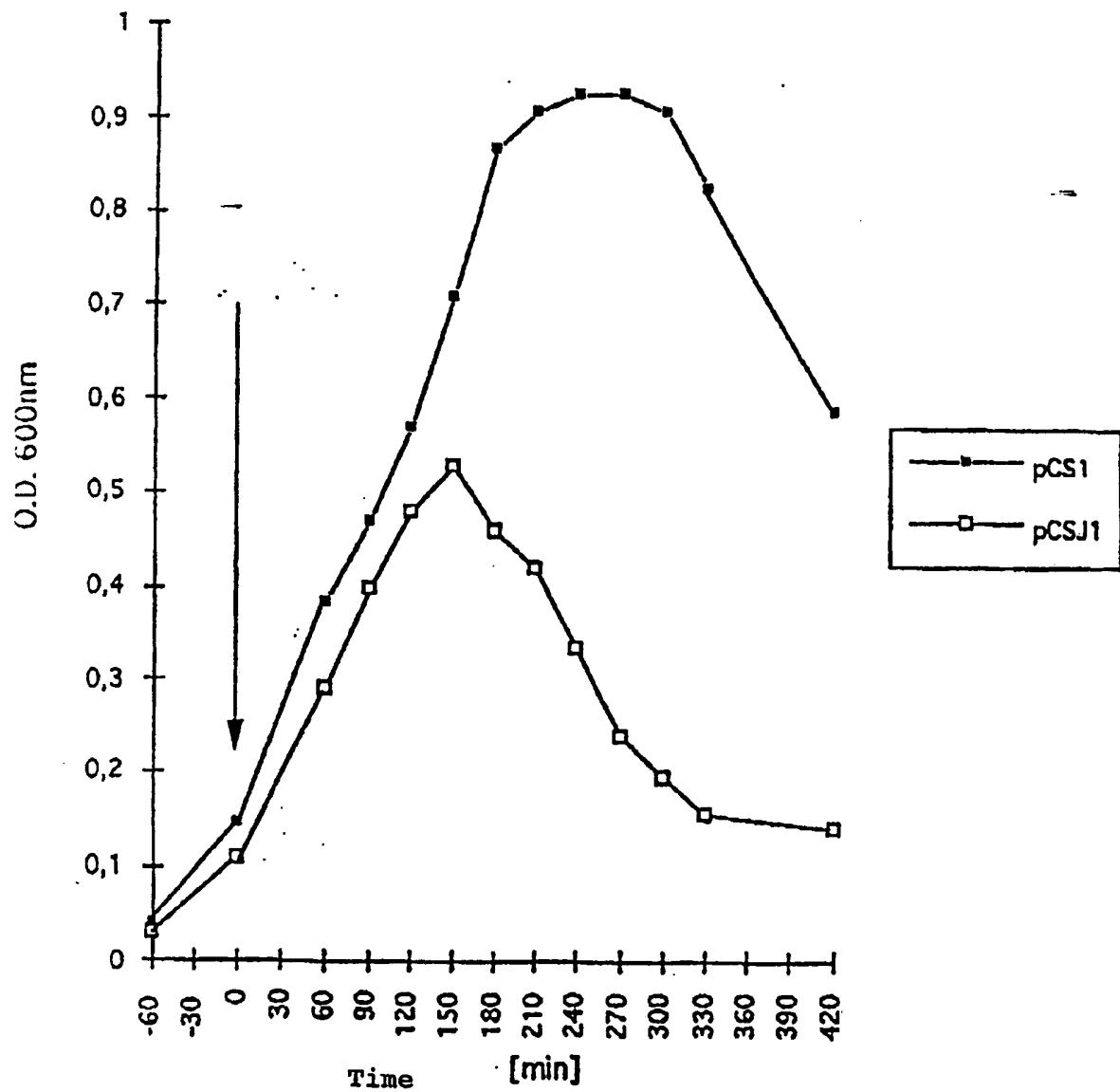


Fig. 5: Growth of *E. coli* MC4100 (pCS1) and MC4100 (pCSJ1) when the temperature is changed from 37°C to 28°C (↓)

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Fig. 6a

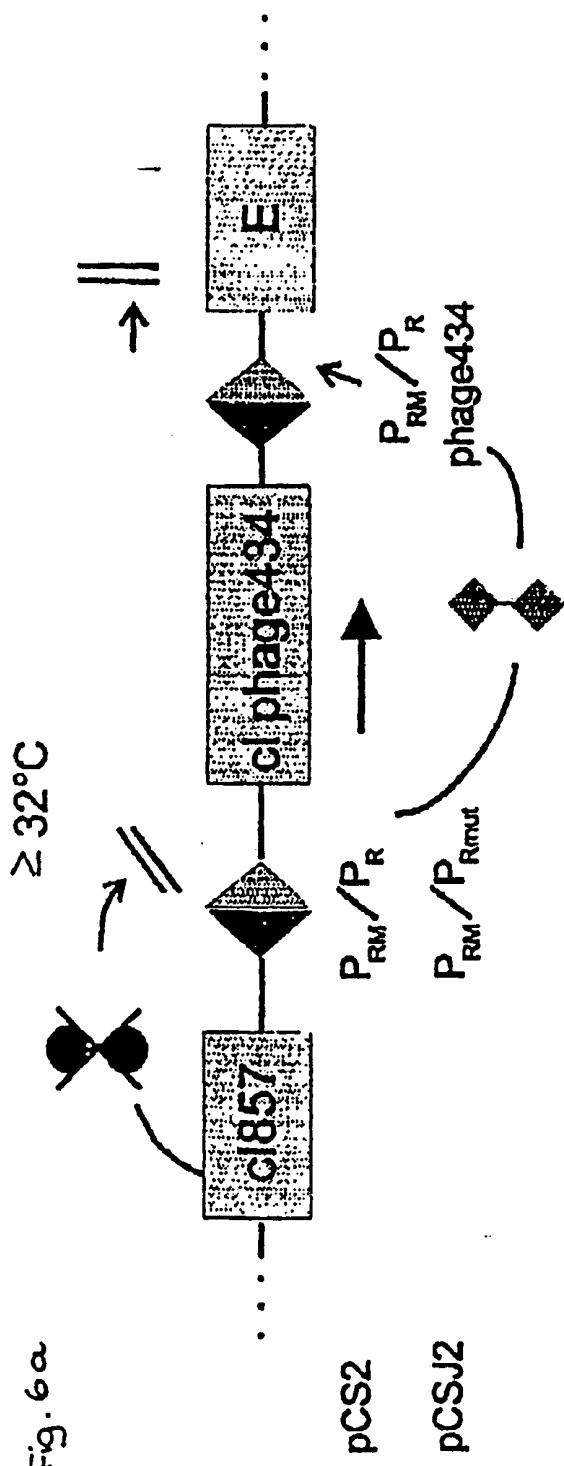


Fig. 6b

